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How Calmodulin Interacts with the Adenosine A_{2A} and the Dopamine D₂ Receptors

Amina S. Woods^{*,†}, Daniel Marcellino[‡], Shelley N. Jackson[†], Rafael Franco^{||}, Sergi Ferré[†], Luigi F. Agnati[§], and Kjell Fuxe[‡]

[†]National Institute on Drug Abuse, IRP, NIH, DHHS, Baltimore, Maryland 21224

[‡]Department of Neuroscience, Karolinska Institute, 17177 Stockholm, Sweden

^{||}Department of Biochemistry, University of Barcelona, Barcelona, Catalunya, Spain

[§]Department of Biomedical Sciences, University of Modena, 411 00 Modena, Italy

Abstract

Receptor heteromerization is a mechanism used by G protein-coupled receptors to diversify their properties and function. We previously demonstrated that these interactions occur through salt bridge formation between epitopes of the involved receptors. Recent studies claim that calmodulin (CaM) binds to an Arg rich epitope located in the amino-terminus of the dopamine D₂ receptor third intracellular loop. This is the same epitope involved in adenosine A_{2A}-D₂ receptor heteromerization, through Coulombic interaction between the Arg residues and a phosphorylated serine (pS) located in the medial segment of the C-terminus of the A_{2A} receptor. Mass spectrometric analysis indicates that an electrostatic interaction involving the D₂ receptor Arg-rich epitope and several CaM acidic epitopes are mainly responsible for the D₂ receptor-CaM binding. CaM could also form multiple noncovalent complexes by means of electrostatic interactions with an epitope localized in the proximal segment of the C-terminus of the A_{2A} receptor. Ca²⁺ disrupted the binding of CaM to the D₂ but not to the A_{2A} receptor epitope, and CaM disrupted the electrostatic interactions between the D₂ receptor epitope and the more distal A_{2A} receptor epitope. A model is introduced with the possible functional implications of A_{2A}-D₂-CaM interactions. These *in vitro* findings imply a possible regulatory role for CaM in receptor heteromers formation.

Keywords

calmodulin; dopamine D₂ receptor; adenosine A_{2A} receptor; receptor heteromers; mass spectrometry

Introduction

Receptor heteromerization constitutes a common mechanism utilized by G protein-coupled receptors to diversify their properties and function.¹⁻³ Thus, receptor heteromers constitute functional entities that possess different biochemical characteristics with respect to the individual components of the heteromer and function as processors of computations that modulate cell signaling.^{2,3} Each receptor in the complex displays its characteristic properties, which could be affected by the presence of the other receptor or on the simultaneous stimulation of both receptors in the heteromer. An extensively studied

* To whom correspondence should be addressed. Amina S. Woods, National Institute on Drug Abuse, IRP, NIH, DHHS, 333 Cassell Drive, Baltimore, MD 21224. Tel: 443-740-2747. E-mail: awoods@intra.nida.nih.gov.

heteromer is that of adenosine A_{2A} and dopamine D₂ receptors. A_{2A}-D₂ receptor heteromers are localized in the GABAergic striatal efferent neurons and provide a new target for antiparkinsonian drugs.^{2,4} We previously mapped some of the key motifs involved in the protein-protein interactions responsible for A_{2A}-D₂ receptor heteromerization and found that an Arg (R)-rich domain located in the N-terminal segment of the long third intracellular loop (NI3L) of the D₂ receptor forms a noncovalent complex (NCX) with a phosphorylated serine (pS) residue located in the long C-terminus of the A_{2A} receptor,^{5,6} through a strong electrostatic interaction. We previously demonstrated that the arginine-phosphate electrostatic interaction possesses a “covalent-like” stability and that bonds formed by this interaction can withstand fragmentation by mass spectrometric collision-induced dissociation at energies similar to those that fragment covalent bonds. We also highlighted the importance of phosphorylation-dephosphorylation events in the modulation of this electrostatic attraction. Phosphorylation of the acidic epitope, a casein kinase one consensus site, makes it available to interact with the basic epitope. On the other hand, phosphorylation of serine and/or threonine residues adjacent to the basic epitope, a protein kinase A consensus site, slows down the attraction between the epitopes. The same kind of electrostatic interaction has been shown to be fundamental for the heteromerization of dopamine D₁ and NMDA receptors, involving a pS located in the tail of the D₁ receptor and an Arg-rich epitope located in the C-terminus of the NR1-1 subunit of the NMDA receptor.⁶⁻⁹ Although analyzed here in the frame of receptor heteromerization, the arginine-phosphate electrostatic interaction most likely represents a general mechanism in protein-protein interactions.

Calmodulin (CaM) is a small (17 kDa), highly conserved, soluble, intracellular, acidic Ca²⁺-binding protein (Figure 1a and b) that is considered to be a major transducer of Ca²⁺ information in the cell.¹⁰ It binds and therefore regulates in a Ca²⁺-dependent manner many different proteins, including ionotropic receptors¹¹ and several Gprotein-coupled receptors.¹²⁻¹⁹ Several studies have shown that CaM also binds to the same Arg-rich domain of the D₂ and NMDA receptors involved in A_{2A}-D₂ and D₁-NMDA receptor heteromerization.^{11,13,19} Stimulation of D₁ receptor results in disruption of its binding to the Arg-rich domain of the NR1-1 subunit, decreasing D₁-NMDA receptor heteromerization and allowing the recruitment of CaM, which then seems to also bind and promote the activation of PI-3 kinase.⁹ Binding of CaM to the Arg-rich domain of the D₂ receptor, which has also been shown to be involved in D₂ receptor-mediated G_{i/o} (Guanine nucleotide binding proteins a family of proteins involved in second messenger cascades) activation,²⁰ has been reported to reduce receptor signaling through adenylyl-cyclase.¹³ Therefore, occupation of the Arg-rich domain of the D₂ receptor seems to reduce G_{i/o}-dependent signaling. In fact, A_{2A}-D₂ receptor heteromers seem to signal preferentially through the G_q-PLC pathway.⁴ It would, therefore, be important to determine to what extent CaM modulates A_{2A}-D₂ receptor heteromerization. Recently, we have also found that 2 R separated by an isoleucine (I) or proline (P) residues (**RIR** and **RPR**) can also establish electrostatic interactions with acidic motifs (in preparation).

Previous studies have suggested that the binding sites of CaM in different proteins are often formed by amphiphilic domains, with hydrophobic residues interspersed with several positively charged ones.¹⁰ The same properties were suggested to determine the ability of the Arg-rich domain of the D₂ receptor to bind CaM.^{13,19} In the present mass spectrometric study, we first demonstrate that an electrostatic interaction is probably responsible for the CaM-D₂ receptor interaction. We then evaluated the possibility that CaM could disrupt the strong epitope-epitope electrostatic interaction involved in A_{2A}-D₂ receptor heteromerization. In addition, we analyzed the possibility of CaM-A_{2A} receptor interactions. We introduce a heuristic model of CaM-A_{2A} receptor-D₂ receptor interactions

which suggests that CaM plays a key role in the dynamics of A_{2A}-D₂ receptor heteromerization and the signaling of A_{2A}-D₂ receptor heteromers.

Material and Methods

Peptides

Recombinant bovine CaM (MW = 16 707.2) expressed in *Escherichia coli* was purchased from Millipore and reconstituted in water. All peptides, **VLRRRRKRVN** (D₂ NI3L; epitope from the N3IL of the human D₂ receptor; MW = 1352.7), **VLAAAAKAVN** (mD₂ NI3L; modified D₂R epitope, where all R are replaced by alanine, A, residues; MW = 927.1), **RIREFRQTFR** (A_{2A}-1; epitope from the N-terminal segment of the C-terminus of the human A_{2A} receptor, MW = 1408.6), and **SAQEpSQGNT** (A_{2A}-2; phosphorylated epitope from the medial segment of the C-terminus of the human A_{2A} receptor, where pS denotes a phosphorylated Serine, MW = 1000.9), were synthesized at the Johns Hopkins School of Medicine Peptide Synthesis Core Facility. Stock solutions of each peptide were prepared in water at a concentration of 1 nmol/μL. Sample mixtures, consisting of an equal volume of CaM at 30 pmol/μL and **RIREFRQTFR**, **VLRRRRKRVN**, **VLAAAAKAVN**, or **VLRRRRKRVN** + **SAQEpSQGNT** at 30 pmol/μL were used for mass analysis. When CaCl₂ was added, it was to a final concentration of 10 mg/dL.

Chymotryptic Digests

Enzymatic digestions were performed by combining 2 μL of CaM solution or CaM+peptide solution, 4 μL of ammonium bicarbonate (25 mM, pH 8.0), and 2 μL of chymotrypsin (0.5 μg/μL). Chymotrypsin was purchased from Roche Molecular Biochemicals (Indianapolis, IN). The enzyme was used at a concentration of 0.5 μg/μL. A 0.3 μL aliquot portion of the digest was deposited on the sample plate, and the reaction was stopped at time intervals of 1, 2, 3, and 5 min by addition of 0.3 μL matrix. Blank digests were also evaluated, where water was substituted for the peptides.²¹⁻²³

Mass Spectrometer

A MALDI-TOF/TOF (4700 Proteomics Analyzer, Applied Biosystems, Framingham, MA) was used in this work for both MS and MS/MS analysis in positive and negative ion mode and has been previously described.⁶ A Nd: YAG laser (355 nm) at a repetition rate of 200 Hz was employed for ionization. For MS analysis, mass spectra were the sum of 400 laser shots and acquired in linear mode.

Matrix

2,6-Dihydroxyacetophenone, DHA, (Fluka, Buchs, Switzerland) was prepared fresh daily as a saturated solution in 50% ethanol.

Molecular Modeling

The CaM was modeled by Chem 3D Pro Version 10 (CambridgeSoft, Cambridge, MA). The model was energy minimized using the MM2 force field with a minimum rms gradient of 0.100.

Results

Binding of CaM to the Arg-Rich Epitope of the D₂ Receptor

We have previously shown, using pull down experiments, mass spectrometry, and BRET, that an electrostatic interaction between the Arg-rich epitope of the N3IL of the D₂ receptor (D₂NI3L; sequence ₂₁₅**VLRRRRKRVN**₂₂₄ of the human D₂ receptor) and a

phosphorylated Ser residue located in the long C-terminus of the A_{2A} receptor (A_{2A}-2; sequence **SAQEpSQGNT**₃₇₀₋₃₇₈ in the human A_{2A} receptor) are involved in A_{2A}-D₂ receptor heteromerization. 5-7 CaM contains several epitopes that could also form NCX with the D₂ NI3L. Four epitopes in particular [**5TEEQ**_{8, 80}, **DSEEEI**_{85, 117}, **TDEEVDEM**_{124, 137}, **NYEEFV**₁₄₂] fit the pattern we observed previously; mainly that the acidic epitopes have a Ser/thr residue upstream or downstream from the acidic residues in the epitope and the consensus site for the phosphorylating enzyme is that of casein kinase. In fact, D₂ NI3L formed multiple noncovalent complexes (NCX) with the Glu (E)- and Asp (D)-rich epitopes of CaM. The spectrum in Figure 2a shows molecular ions (MH⁺) at amu 16 707.2 (CaM), 18 059.9 (CaM+ 1 D₂ NI3L), 19 412.2 (CaM+2 D₂ NI3L), and 20 765.3 (CaM+3 D₂ NI3L). Thus, as many as three copies of the epitope can add to CaM, supporting the multiplicity of available acidic sites of CaM that can interact with the D₂ receptor epitope, through electrostatic attraction between the positive delocalized charge of the Arg guanidinium groups and the negative delocalized charge of Glu and Asp carboxyl groups. The mixture of CaM + modified D₂ NI3L (VLAAAKAVN) did not result in complex formation (Figure 2b), demonstrating the predominant electrostatic character of the CaM-D₂ receptor interaction. If CaM is added to the D₂R epitope (**215VLRRRRKRVN**₂₂₄) and the A_{2A} acidic epitope (SAQEpSQGNT), the NCX that is usually formed between the A_{2A} acidic epitope and D₂ epitope (MH⁺ 2354.1) is disrupted, regardless of whether CaM is added before or after the NCX is formed. The spectrum in Figure 2c shows that the 2354.1 peak is absent while the CaM-D₂R NCX are present at *m/z* (18 059.1 and 19 411.9). This finding is surprising from the point of view of mass spectrometry, MH⁺ of NCX of peptides are far easier to detect than those of a peptide and a protein.

Binding of CaM to an Epitope from the C-Terminus of the A_{2A} Receptor

Because we were interested in studying the possible interactions between CaM and the A_{2A}-D₂ receptor heteromer, and in view of the increasing list of GPCRs that bind CaM (see Introduction), it was also important to investigate the possible direct interaction between CaM and the A_{2A} receptor. A search in the literature (PubMed, NLM, NIH) did not provide any previously reported evidence. However, introducing the A_{2A} receptor sequence in the Internet-based search program "Calmodulin Target Data Base" (Cancer Institute, Ontario, 2002; <http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html>) disclosed the existence of a very likely binding site in the N-terminal segment of the C-terminus of the A_{2A} receptor. This epitope (A_{2A}-1), sequence **291RIREFRQTFR**₃₀₀ in the human A_{2A} receptor, also contains several Arg residues. A brief perusal of the A_{2A}-1 epitope sequence shows that as in the case of the D₂R epitope, the A_{2A}-1 epitope contains a Thr, in a PKC/PKA consensus site. Hence *phosphorylation/dephosphorylation* of that residue might play a physiological role. In addition to the **RIR** motif, which forms stable NCX with adjacent acidic residues, phosphate, and adjacent aromatic residues, the presence of the **FR** motif further increases the stability of the interaction, resulting in CaM forming multiple stable NCX with A_{2A}-1. The spectrum in Figure 2d shows MH⁺ at amu 16 707.2, 18 115.8 (CaM+1 A_{2A}NC), 19 524.4 (CaM+2 A_{2A}NC), 20 933.0 (CaM+3 A_{2A}NC), and 22 341.6 (CaM+4 A_{2A}NC).

Enzymatic Digest of NCX between CaM and D₂ and CaM and A_{2A} Receptor Epitopes

We have previously shown that the residues involved in NCX formation are protected from the reach of enzymes, and hence the interaction sites are not digested.²¹⁻²³ The chymotryptic digest of CaM (Figure 3a), CaM+**215VLRRRRKRVN**₂₂₄ (Figure 3b), and CaM+**291RIREFRQTFR**₃₀₀ (Figure 3c) yielded the data seen in Figure 3a-c. The fragments generated from the NCX digests are in Tables 1 and 2. Perusal of the fragments shows that NCX made of the acidic CaM epitopes **80DSEEEI**_{85, 117}, **TDEEVDEM**₁₂₄, and **137NYEEFV**₁₄₂ and basic peptide fragments were present after chymotryptic digestion

of CaM+D₂ NI3L and CaM+A_{2A}-1, supporting their involvement in protein–protein interactions between CaM and D₂ and A_{2A} receptors. Furthermore, after enzymatic digest of the CaM+A_{2A}-1 complexes the A_{2A}-1 epitope always kept the first 5 residues, suggesting that indeed **RIR** are the residues involved in the interactions with CaM.

Role of Ca²⁺ in the Interactions between CaM and the D₂ and A_{2A} Receptor Epitopes

Addition of CaCl₂ to the sample solutions for a final concentration of 10 mg/dL yielded the following results. When added to CaM+₂₁₅**VLRRRRKR**VN₂₂₄, CaCl₂ significantly disrupted NCX formation, as the relative abundance of both 18 059.9 (CaM+1 D₂ NI3L) and 19 412.2 (CaM+2 D₂ NI3L) is about a fifth that with no CaCl₂ added (Figures 2a and 4a). However, in the case of CaM+A_{2A}-1, addition of the same amount of CaCl₂ did not have any effect on NCX formation, as seen in Figure 4b, where spectra of the NCX without and with CaCl₂ are identical. We also added the same amount of CaCl₂ to a mixture of the D₂ NI3L and A_{2A}-2 epitopes and detected no effect on formation of the NCX at *m/z* 2354.1, as displayed in Figure 4c.

Discussion

Scientists in fields as diverse as Neuroscience and Biophysics are trying to understand and make sense of the physical interactions involved in receptor heteromerization. We believe that proteomics is the tool of choice for defining receptor heteromers as well as other proteins interacting with the heteromers at the molecular level, as only through a comprehensive understanding of the receptors protein structure can we get to the macromolecular basis of receptor heteromerization.^{6,20,23-25} These epitope–epitope electrostatic interactions have been shown to be involved in heteromerization of A_{2A} and D₂ receptors and NMDA and D₁ receptors (see Introduction).

Previous studies have shown that CaM binds to the same Arg-rich epitopes of the D₂ and NMDA receptors involved in A_{2A}-D₂ and D₁-NMDA heteromerization.^{11,13,19} The Arg-rich epitope of the D₂ receptor is localized in the N3IL, which contains multiple positively charged residues (mostly R) and several hydrophobic residues. The amphiphilic character with apolar hydrophobic residues and polar positively charged residues has been suggested to be the main characteristic of CaM binding sites.¹⁰ By studying the effects of different mutated D₂ receptors, Liu et al. suggested that the hydrophobic residues I₂₁₀, L₂₁₆, and V₂₂₃ were determinant for CaM binding.¹⁹ Our data, instead, indicate that the adjacent R are mainly responsible for CaM binding. First, I₂₁₀ is not included in the D₂ receptor epitope used in our experiments (D₂NI3L; ₂₁₅**VLRRRRKR**VN₂₂₄), which forms multiple NCX with CaM. Second, the mixture of CaM+**VLAAAKAV**N did not result in any NCX with CaM. The disruptive role of the mutations in the experiments by Liu et al. can be explained by the fact that aliphatic amino acids were replaced by cysteine, which is a very interactive amino acid residue with an available pair of electrons on its sulfur.

CaM amino acid composition shows that 32.5% of its residues contain a negative charge. Furthermore, CaM has several casein kinase consensus sites located in the vicinity of acidic residues; hence CaM is even more acidic *in vivo*, as it can be phosphorylated (*E. coli* CaM is not phosphorylated). Previous studies already suggested that acidic domains of CaM are involved in interactions with positively charged epitopes of other proteins. O’Neil and DeGrado concluded that when considering peptides that bind to CaM, “very basic amphiphilic peptide with a large cross-sectional area might be inferred from consideration of the protein’s electrostatic surface”.²⁶ Also, Andre et al. demonstrated that CaM binds R-rich peptides from a myosin light chain kinase²⁷ and Pate et al. found that peptides from voltage dependent Ca²⁺ channels that bind to CaM also have basic residues.²⁸ Our data strongly support that the CaM binding to the D₂ receptor R is mainly through salt bridge formation

between D₂ receptor basic residues and CaM acidic residues. The enzymatic digest experiments suggest that the very acidic epitopes **80DSEEEI_{85, 117}TDEEVDEM₁₂₄**, and **137NYEEFV₁₄₂** are particularly involved in CaM–D₂ receptor interactions.

For the first time, evidence is also provided for the binding of CaM to an epitope of the A_{2A} receptor, located in the most proximal segment of its long C-terminus. The epitope (A_{2A}–1; **291RIRFRQTFR₃₀₀**) fulfills the known main characteristic of CaM binding sites, with several R (positively charged residues) and I and F (hydrophobic residues). Again, our data support the predominant role of electrostatic interactions in CaM–A_{2A} receptor epitope interactions. As mentioned before, the **RIR** motif establishes electrostatic interactions with adjacent acidic residues or phosphate groups. Furthermore, the enzymatic digest experiments also support the involvement of the very acidic epitopes of CaM in its interactions with the A_{2A} receptor epitope.

The electrostatic interactions of CaM with the D₂ and the A_{2A} receptor epitopes can have important implications for the function of A_{2A}–D₂ receptor heteromers. First of all, CaM can bind simultaneously to both A_{2A} and D₂ receptors, and therefore, A_{2A} receptors can use direct CaM-independent and indirect CaM-dependent mechanisms to establish intermolecular interactions with the D₂ receptor. In other words, CaM can provide an indirect mechanism, a linker for A_{2A} and D₂ receptor interactions. The experiments of competition between CaM and the A_{2A}–2 epitope for the D₂NI3L epitope indicate that in the presence of CaM, the CaM-dependent mechanism predominates. However, this mechanism becomes less important when CaM binds Ca²⁺. Thus, an important difference between the interactions between CaM with D₂ and A_{2A} receptor epitopes was their regulation by Ca²⁺. In most instances Ca²⁺ binding to CaM increases its binding to the interacting protein.¹⁰ Our experiments indicate that Ca²⁺ does not modulate the interaction of CaM with the A_{2A} receptor epitope and that it significantly decreases its interaction with the D₂ receptor epitope.

Obviously, CaM must play an important role in D₂ receptor signaling and in the modulation of A_{2A}–D₂ receptor heteromerization and the function of the A_{2A}–D₂ receptor heteromers. It has been shown that D₂ receptor couples to G_{i/o}, and therefore negatively to adenylyl-cyclase, when not forming heteromers, and that it couples to G_{q/11}–PLC signaling when forming heteromers with D₁ receptors and most probably with A_{2A} receptors.^{4,29} The inability of D₂ receptors to signal through G_{i/o} proteins when bound to A_{2A} receptors or to CaM^{4,13} is most probably related to the fact that the D₂ NI3L epitope is fundamental for the coupling and signaling to G_{i/o} proteins.²⁰ From the present *in vitro* results we can postulate that, in the cell: (i) CaM binds to the A_{2A} receptor; (ii) D₂ receptors form heteromers with A_{2A} receptors with or without CaM; (iii) Ca²⁺ disrupts CaM-dependent and facilitates CaM-independent A_{2A}–D₂ receptor heteromerization; and (iv) CaM produces a Ca²⁺-dependent switch from G_{q/11}–PLC to G_{i/o}–adenylylcyclase signaling in D₂ receptors not forming heteromers or forming heteromers with A_{2A} receptors (Figure 5). Experiments are in progress to test these hypotheses.

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(a) 1 ADQL**TEE**QIA EFKEAFSLFD KDGDGTITTK ELGTVMRSLG QNP**TEA**ELQD 50
 51 MINEVD**DAD**GN GT**IDF**PEFLT MMARKMK **DTD** **SEEE**IREA**FR** VFDKDGNGYI 100
 101SAAELRHVMT NLGEKL**TDEE** **VDEM**IREADI **DGDG**QV**NYEE** **FVQM**MTAK 148

5**TEE**Q₈, 80**DSEEE**I₈₅, 117**TDEE**VDEM₁₂₄, 137**NYEE**FV₁₄₂

Figure 1.

(a) Bovine CaM sequence and (b) bovine CaM model.

Figure 2.

Binding of the D₂ NI3L and the A_{2A}-1 epitopes to CaM. Spectra of (a) NCX of CaM and the D₂ NI3L epitope (VLRRRRKRVN). (b) No NCX form between CaM and the modified D₂ NI3L epitope (VLAAAKAVN) where Arg were replaced by Ala. (c) Disruption of the interaction of the D₂ NI3L epitope (VLRRRRKRVN) and the A_{2A}-2 epitope (SAQEpSQGNT) by CaM. Although both peptides are present, no NCX forms between these epitopes in the presence of CaM. (d) Mixture of CaM and the A_{2A}-1 epitope (RIREFRQTFR).

Figure 3.

Fragments generated by the chymotryptic digest of CaM, the NCX of CaM with the D₂ NI3L and the A_{2A}-1 epitopes. Spectra of enzymatic digest of (a) CaM alone, (b) mixture of CaM and the D₂ NI3L epitope (VLRRRRKRVN), and (c) mixture of CaM and the A_{2A}-1 epitope (RIREFRQTFR). * denotes CaM fragments.

Figure 5.

Scheme of hypothetical functional interactions between CaM and A_{2A} and D₂ receptors. (a) In the absence of CaM, A_{2A} receptor binds to the D₂ receptor by an electrostatic interactions between the A_{2A}-2 epitope (SAQEpSQGNT), localized in the medium segment of the C-terminus of the A_{2A} receptor (pink box), and the D₂NI3L epitope (VLRRRRKRNVN), localized in the N-terminal segment of the third intracellular loop of the D₂ receptor (blue box). (b) A_{2A}-D₂ receptor heteromerization could also involve CaM as a linker, by its ability to simultaneously bind to the D₂NI3L epitope and to the A_{2A}-1 epitope (RIREFRQTFR), localized in the proximal segment of the C-terminus of the A_{2A} receptor (red box). (c-d) Ca²⁺ (black dots) disrupts the binding of CaM to the D₂ receptor but not to

the A_{2A} receptor, which leaves the D₂NI3L epitope to interact with the A_{2A}-2 epitope or with G_{i/o} proteins (see text).

Table 1Fragments Generated from Chymotryptic Digest of CaM+VLRRRRKRVN (D₂NI3L)

CaM frg + D ₂ NI3L frg	CaM frg MW	D ₂ NI3L frg MW	MH ⁺ observed
57–103 + 217–224	5287.5	1140.7	6429.8
67–112 + 217–224	5393.6	1140.7	6535.3
42–93 + 217–224	6009.7	1140.7	7151.1
84–137 + 217–224	6108.9	1140.7	7250.5
64–119 + 217–224	6410.1	1140.7	7551.7
59–116 + 217–224	6622.2	1140.7	7753.9
67–131 + 217–224	7581.6	1140.7	8723.3
57–126 + 217–224	8025.8	1140.7	9167.5
8–86 + 217–224	8859.3	1140.7	10001.0

Table 2Fragments Generated from Chymotryptic Digest of CaM+RIREFRQTFR (A_{2A}-1)

CaM frgm + A _{2A} -1 frg	CaM frg MW	A _{2A} -1 frg MW	MH ⁺ observed
39-95 + 291-295	6551.0	719.4	7271.1
64-109 + 291-295	6997.2	719.4	7717.6
66-126 + 291-295	7135.4	719.4	7856.2
68-129 + 291-295	7224.4	719.4	7944.9
69-135 + 291-295	7661.6	719.4	8381.7
47-116 + 291-295	7994.8	719.4	8714.8
68-138 + 291-295	8185.8	719.4	8906.9
44-124 + 291-295	9244.3	719.4	9964.1
62-146 + 291-295	9883.6	719.4	10603.4
38-142 + 291-295	11890.5	719.4	12611.0